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ORIGINAL ARTICLE

A three–decade survey of Brazilian *Fusarium oxysporum* f. sp. *lycopersici* races assessed by pathogenicity tests on differential tomato accessions and by molecular markers

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Abstract

Aim: Physiological race determination of 143 *Fusarium oxysporum* f. sp. *lycopersici* (FOL) isolates collected along 30 years in major tomato-producing regions of Brazil.

Materials and Results: Physiological races were determined via root-dipping inoculation of differential tomato accessions and by the PCR-based marker system of Hirano and Arie (2006). According to pathogenicity/virulence assays, five race 1, 23 race 2 and 115 race 3 isolates were identified. FOL race 1 and 2 isolates prevailed up to early 2000s. Afterwards, the large majority of the isolates was classified as the invasive race 3. Novel reports of race 3 were done in five states, thus expanding its geographical distribution. Using this PCR-based marker system, a precise discrimination was observed for all race 3 isolates. However, all race 1 and 2 isolates displayed only the cosmopolitan race 1–specific amplicon pattern.

Conclusion: The development and/or validation of novel race-specific marker systems are necessary to allow a precise discrimination of the potentially endemic Brazilian FOL race 2.

Significance and Impact of the Study: The present characterization of isolates indicates that distinct evolutionary mechanisms are acting to select new FOL races and/or genetic variants across agroecosystems around the globe.

Introduction

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) Snyder and Hansen (FOL) is the causal agent of vascular wilt in tomatoes (*Solanum lycopersicum* L.), which is one of the major diseases affecting this crop across tropical and subtropical regions of the world (McGovern 2015). FOL is more adapted to warm regions and it is a typical vascular wilt-inducing fungus, being able to invade the host root system and colonize the xylem vessels (Srinivas *et al.* 2019). Three physiological races of FOL were described, being defined according to their ability to infect a set of tomato (*Solanum* section *Lycopersicon*) accessions carrying distinct resistance factors (Grattidge and O'Brien 1982). A repertoire of four dominant resistance factors (*I*, *I–2*, *I–3* and *I–7*) has been characterized in distinct tomato accessions (Gonzalez-Cendales *et al.* 2016; Catanzariti *et al.* 2017). The *I–2* gene from *S. pimpinellifolium* (located on chromosome 11) controls resistance to race 1 and race 2 isolates, displaying in its structure the nucleotide binding and leucine-rich repeat (LRR) motifs (Simons *et al.* 1998). The race 1–specific *I* gene was introgressed from *S. pimpinellifolium* (also located on chromosome 11) and it encodes an atypical membrane-anchored, LRR receptor-like protein (Catanzariti *et al.* 2017). The FOL race 3 resistance gene *I–3* from *S. pennellii* is located on chromosome 7. The *I–3* gene encodes one S–receptor–like kinase and controls resistance to race

2 and race 3 isolates (Catanzariti *et al.* 2015). The *I–7* gene from *S. pennellii* (located on chromosome 8) encodes an LRR receptor-like protein and displays a wide-spectrum resistance, being effective against all FOL races (Gonzalez-Cendales *et al.* 2016).

Races 1 and 2 are the most disseminated FOL variants, being present in virtually all major tomato-producing areas around the world (Stravato *et al.* 1999; Kuramae and Souza 2002; Sibounnavong *et al.* 2012; Debbie *et al.* 2018). However, the geographical distribution of FOL race 3 isolates is dramatically expanding in recent years, being already reported in Australia (Grattidge and O'brien 1982); United States (Volin and Jones 1982; Davis *et al.* 1988); Mexico (Valenzuela-Ureta *et al.* 1996; Holguín-Peña 2005); Turkey (Baysal *et al.* 2009); South Korea (Choi *et al.* 2013); South Africa (Jacobs *et al.* 2013); Chile (Sepúlveda-Chavera *et al.* 2014); Algeria (Debbie *et al.* 2018); China (Chang *et al.* 2018; Ye *et al.* 2020) and Argentina (Malbrán *et al.* 2020). The recent invasion of FOL race 3 isolates was also detected in three Brazilian states: Espírito Santo (Reis *et al.* 2005), Rio de Janeiro (Reis and Boiteux 2007) and Bahia (Barboza *et al.* 2013).

Precise race determination of FOL isolates under field conditions and in association with tomato seeds and seedlings are important pieces of information in the management of this disease (Inami *et al.* 2010). A very efficient and reliable molecular marker system for race-specific identification of FOL isolates was developed by Hirano and Arie (2006), which is based upon nucleotide polymorphisms in genes encoding cell-wall-degrading enzymes. This marker system has been capable of not only discriminating the three FOL races but also *F. oxysporum* f. sp. *radicis–lycopersici* (FORL) isolates across distinct continents (Hirano and Arie 2006; Balogun *et al.* 2008; Baysal *et al* 2009; Çolak and Biçici 2013; Chang *et al.* 2018; Cabral *et al.* 2020; Ye *et al.* 2020).

Thus far, the works dealing with the characterization of FOL physiological races in Brazil are limited to pathogenicity assays with a small number of isolates (Reis *et al.* 2005; Reis and Boiteux 2007; Barboza *et al.* 2013), with only a reduced amount of works employing more refined and informative molecular characterization (Kuramae and Souza 2002; Amaral *et al.* 2013). In fact, extensive surveys and diversity analyses of FOL isolates combining pathogenicity tests and molecular marker assays are scarce across South America. In this scenario, the objective of the present work was to characterize a collection of FOL isolates (obtained along three decades in geographically distinct areas) by combining pathogenicity tests in race-differential tomato accessions and also via the worldwide validated molecular marker system described by Hirano and Arie (2006). To our knowledge, the present work represents the most comprehensive analysis of the race variability of FOL isolates associated with tomato vascular wilt in Neotropical areas.

Materials and methods

Collection of FOL isolates

In all, 143 isolates in association with vascular symptoms were collected from 1990 to 2019 across major tomato-producing regions (Table 1). This set of isolates is preserved in the collection of plant pathogenic fungi of the Plant Pathology Laboratory located at the Embrapa Hortaliças. Field isolates were initially purified from diseased xylem and subsequently employed in inoculation assays (see section below), employing the cultivar 'Ponderosa' (susceptible to all races). Each fungal isolate was then re-isolated on potato dextrose agar medium supplemented with tetracycline (PDA-t/50 μg ml^{-1}) and stored at 10°C in water as described by Castellani (1963) and also by cryopreservation at −80°C in a deep freezer. Morphological and cultural characteristics of each individual isolate were observed and compared with literature descriptions (Leslie and Summerell 2006) to confirm their classification as *F. oxysporum* isolates. For pathogenicity assays, the isolates were recovered from the storage by transferring their mycelia to PDA-t and maintaining them in BOD growth chambers at 25°C, 12 h light for 7 days (Cabral *et al.* 2018).

Reaction of the differential tomato accessions to each individual FOL isolate

Physiological race identity of all 143 isolates was determined by root-dipping inoculation of seedlings of the following set of five FOL differential tomato accessions: *S. lycopersicum* 'Ponderosa' (susceptible to all races), *S. lycopersicum* 'Viradoro' (resistant to race 1 due to the presence of the gene *I*) (Giordano *et al.* 2000), *S. lycopersicum* 'Floradade' (resistant to races 1 and 2 due to the presence of the *I* and *I–2* genes), *S. pennellii* 'LA 716' (resistant to races 2 and 3 due to the presence of the gene *I–3*) and *S. lycopersicum* 'BHRS-2,3' (resistant to all the three races conditioned by the locus *I–7*). Conidia were produced in potato dextrose under standard conditions (12 h of light and 25°C ± 2°) for 7 days. The conidial suspension was filtered and adjusted to 1×10^6 conidia per ml. The seeds of the differential tomato accessions were sown in trays with 128 cells (one seed per cell), filled with sterile substrate (Plantmax®). Plants with the first two pairs of true leaves fully open (about 21 days after planting) were removed from the cells with a gentle spray of water, aiming to preserve root integrity. The root

Table 1 Physiological race characterization of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) isolates obtained from tomato (*Solanum lycopersicum* L.) in Brazil by pathogenicity assay with a set of differential *Solanum* (section *Lycopersicon*) accessions and by PCR assay using prime pairs of the molecular marker system described by Hirano and Arie (2006). Table fields with grey colour are indicating isolates with observed discrepancies between the pathogenicity and the PCR assays

				Prime pairs of the molecular marker system described by Hirano and Arie (2006)				
FOL Isolate	Collection place in Brazil (City and State)‡	Year	Physiol. race*	Uni	Sp13	Sp23	Spr1	Race†
Fus-023	Belém S. Francisco –PE	1990	2	+	+	–	–	1
Fus-024	Belém S. Francisco –PE	1991	2	+	+	–	–	1
Fus-025	Belém S. Francisco –PE	1991	2	+	+	–	–	1
Fus-026	Bezerros–PE	1991	2	+	+	–	–	1
Fus-027	Botucatu–SP	1992	1	+	+	–	–	1
Fus-028	Botucatu–SP	1992	2	+	+	–	–	1
Fus-029	Uberlândia–MG	1992	1	+	+	–	–	1
Fus-030	Uberlândia–MG	1992	1	+	+	–	–	1
Fus-290	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-291	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-292	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-295	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-296	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-297	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-298	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-299	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-300	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-301	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-302	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-303	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-304	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-305	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-306	Camocim S. Félix-PE	1997	2	+	+	–	–	1
Fus-087	Belém S. Francisco –PE	2002	2	+	+	–	–	1
Fus-088	Belém S. Francisco –PE	2002	2	+	+	–	–	1
Fus-089	V. Nova Imigrante–ES	2003	3	+	+	+	–	3
Fus-090	V. Nova Imigrante–ES	2003	3	+	+	+	–	3
Fus-091	V. Nova Imigrante–ES	2003	3	+	+	+	–	3
Fus-094	V. Nova Imigrante–ES	2004	3	+	+	+	–	3
Fus-112	V. Nova Imigrante–ES	2004	3	+	+	+	–	3
Fus-118	V. Nova Imigrante–ES	2005	3	+	+	+	–	3
Fus-143	V. Nova Imigrante–ES	2005	3	+	+	+	–	3
Fus-145	Muniz Freire–ES	2006	3	+	+	+	–	3
Fus-146	Domingos Martins–ES	2006	3	+	+	+	–	3
Fus-148	Domingos Martins–ES	2006	3	+	+	+	–	3
Fus-149	Domingos Martins–ES	2006	3	+	+	+	–	3
Fus-150	São José de Ubá–RJ	2006	3	+	+	+	–	3
Fus-152	São José de Ubá–RJ	2006	3	+	+	+	–	3
Fus-153	São José de Ubá–RJ	2006	3	+	+	+	–	3
Fus-154	São José de Ubá–RJ	2006	3	+	+	+	–	3
Fus-155	São José de Ubá–RJ	2006	3	+	+	+	–	3
Fus-156	Itaocara–RJ	2006	3	+	+	+	–	3
Fus-167	Brasília–DF	2007	3	+	+	+	–	3
Fus-168	Brasília–DF	2007	3	+	+	+	–	3
Fus-169	Brasília–DF	2007	3	+	+	+	–	3
Fus-175	Paty do Alferes–RJ	2008	3	+	+	+	–	3
Fus-176	Paty do Alferes–RJ	2008	3	+	+	+	–	3
Fus-179	Paty do Alferes–RJ	2009	3	+	+	+	–	3
Fus-180	Paty do Alferes–RJ	2009	3	+	+	+	–	3

(Continued)

Table 1 (Continued)

FOL Isolate	Collection place in Brazil (City and State)‡	Year	Physiol. race*	Prime pairs of the molecular marker system described by Hirano and Arie (2006)				
				Uni	Sp13	Sp23	Spr1	Race†
Fus-181	Domingos Martins–ES	2009	3	+	+	+	–	3
Fus-182	Domingos Martins–ES	2009	3	+	+	+	–	3
Fus-183	Domingos Martins–ES	2009	3	+	+	+	–	3
Fus-188	Domingos Martins–ES	2009	3	+	+	+	–	3
Fus-191	Brasília–DF	2009	3	+	+	+	–	3
Fus-192	Brasília–DF	2009	3	+	+	+	–	3
Fus-204	V. Nova Imigrante–ES	2010	2	+	+	–	–	1
Fus-216	Brasília–DF	2010	3	+	+	+	–	3
Fus-228	Jaguaquara–BA	2011	3	+	+	+	–	3
Fus-229	Jaguaquara–BA	2011	3	+	+	+	–	3
Fus-230	Jaguaquara–BA	2011	3	+	+	+	–	3
Fus-232	Jaguaquara–BA	2011	3	+	+	+	–	3
Fus-233	Jaguaquara–BA	2011	3	+	+	+	–	3
Fus-234	Jaguaquara–BA	2011	3	+	+	+	–	3
Fus-235	Brasília–DF	2011	3	+	+	+	–	3
Fus-256	Coimbra–MG	2012	3	+	+	+	–	3
Fus-257	Poções–BA	2012	3	+	+	+	–	3
Fus-336	Domingos Martins–ES	2012	3	+	+	+	–	3
Fus-258	Brasília–DF	2013	3	+	+	+	–	3
Fus-259	Nova Friburgo–RJ	2013	3	+	+	+	–	3
Fus-260	Castelo–ES	2013	3	+	+	+	–	3
Fus-307	Afonso Cláudio–ES	2012	3	+	+	+	–	3
Fus-308	V. Nova Imigrante–ES	2013	3	+	+	+	–	3
Fus-309	Marechal Floriano–ES	2013	3	+	+	+	–	3
Fus-310	Vargem Alta–ES	2013	3	+	+	+	–	3
Fus-311	Domingos Martins–ES	2013	3	+	+	+	–	3
Fus-312	Domingos Martins–ES	2013	3	+	+	+	–	3
Fus-313	Domingos Martins–ES	2013	3	+	+	+	–	3
Fus-314	Coimbra–MG	2013	3	+	+	+	–	3
Fus-315	Coimbra–MG	2013	3	+	+	+	–	3
Fus-316	Nova Friburgo–RJ	2013	3	+	+	+	–	3
Fus-317	Afonso Cláudio–ES	2013	3	+	+	+	–	3
Fus-318	Afonso Cláudio–ES	2013	3	+	+	+	–	3
Fus-319	Castelo–ES	2013	3	+	+	+	–	3
Fus-320	Paty do Alferes–RJ	2013	3	+	+	+	–	3
Fus-321	Santa Maria Jetibá–ES	2013	3	+	+	+	–	3
Fus-329	Muniz Freire–ES	2013	3	+	+	+	–	3
Fus-330	Coimbra–MG	2013	3	+	+	+	–	3
Fus-331	Nova Friburgo–RJ	2013	3	+	+	+	–	3
Fus-332	Domingos Martins–ES	2013	3	+	+	+	–	3
Fus-335	V. Nova Imigrante–ES	2013	3	+	+	+	–	3
Fus-337	Domingos Martins–ES	2013	3	+	+	+	–	3
Fus-322	Sumidouro–RJ	2014	3	+	+	+	–	3
Fus-323	Sumidouro–RJ	2014	3	+	+	+	–	3
Fus-324	Sumidouro–RJ	2014	3	+	+	+	–	3
Fus-325	Serra–ES	2014	3	+	+	+	–	3
Fus-326	Marilac–MG	2014	3	+	+	+	–	3
Fus-327	Nova Friburgo–RJ	2014	3	+	+	+	–	3
Fus-328	São José de Ubá–RJ	2014	3	+	+	+	–	3
Fus-333	Domingos Martins–ES	2014	3	+	+	+	–	3
Fus-334	São José de Ubá–RJ	2014	3	+	+	+	–	3
Fus-374	Araguari–MG	2015	3	+	+	+	–	3

(Continued)

Table 1 (Continued)

FOL Isolate	Collection place in Brazil (City and State)‡	Year	Physiol. race*	Prime pairs of the molecular marker system described by Hirano and Arie (2006)				
				Uni	Sp13	Sp23	Spr1	Race†
Fus-375	Araguari–MG	2015	3	+	+	+	–	3
Fus-376	Araguari–MG	2015	3	+	+	+	–	3
Fus-377	Goianápolis–GO	2015	3	+	+	+	–	3
Fus-379	Baldim–MG	2015	3	+	+	+	–	3
Fus-380	Baldim–MG	2015	1	+	+	–	–	1
Fus-381	Nerópolis–GO	2015	3	+	+	+	–	3
Fus-382	Nerópolis–GO	2015	3	+	+	+	–	3
Fus-383	Vassouras–RJ	2015	3	+	+	+	–	3
Fus-385	Paty do Alferes–RJ	2015	3	+	+	+	–	3
Fus-388	Paty do Alferes–RJ	2015	3	+	+	+	–	3
Fus-392	Miguel Pereira–RJ	2015	3	+	+	+	–	3
Fus-400	Baldim–MG	2015	3	+	+	+	–	3
Fus-401	Baldim–MG	2015	3	+	+	+	–	3
Fus-402	Goianápolis–GO	2015	3	+	+	+	–	3
Fus-403	Nerópolis–GO	2015	3	+	+	+	–	3
Fus-404	Goianápolis –GO	2015	3	+	+	+	–	3
Fus-424	Jales–SP	2016	3	+	+	+	–	3
Fus-425	Jales–SP	2016	3	+	+	+	–	3
Fus-426	Jales–SP	2016	3	+	+	+	–	3
Fus-439	Paranapoã–SP	2017	3	+	+	+	–	3
Fus-440	Paranapoã–SP	2017	3	+	+	+	–	3
Fus-441	Paranapoã–SP	2017	3	+	+	+	–	3
Fus-442	Paranapoã–SP	2017	3	+	+	+	–	3
Fus-443	Paranapoã–SP	2017	3	+	+	+	–	3
Fus-445	Araguari–MG	2017	3	+	+	+	–	3
Fus-465	Nova Friburgo–RJ	2017	3	+	+	+	–	3
Fus-467	Nova Friburgo–RJ	2017	3	+	+	+	–	3
Fus-470	Nova Friburgo–RJ	2017	3	+	+	+	–	3
Fus-481	Tianguá–CE	2018	3	+	+	+	–	3
Fus-482	Tianguá–CE	2018	3	+	+	+	–	3
Fus-483	Tianguá–CE	2018	3	+	+	+	–	3
Fus-484	Juvenilia–MG	2018	3	+	+	+	–	3
Fus-485	Juvenilia–MG	2018	3	+	+	+	–	3
Fus-486	Juvenilia–MG	2018	3	+	+	+	–	3
Fus-511	Carmópolis–MG	2019	3	+	+	+	–	3
Fus-512	Araguari–MG	2019	3	+	+	+	–	3
Fus-513	Uberlândia–MG	2019	3	+	+	+	–	3
Fus-516	Três Corações–MG	2019	3	+	+	+	–	3
Fus-517	Três Corações–MG	2019	3	+	+	+	–	3
Fus-526	Tianguá–CE	2019	3	+	+	+	–	3
Fus-527	Tianguá–CE	2019	1	+	+	–	–	1
Fus-530	Carnaubal–CE	2019	3	+	+	+	–	3

*Physiological race (1, 2 or 3) as indicated by the reaction of the differential *Solanum* (section *Lycopersicon*) accessions in the root-dipping inoculation assays.

†Race determination (1, 2 or 3) according to the amplicon profile using the molecular marker system developed by Hirano and Arie (2006), where (+) = presence and (–) = absence of the expected PCR amplicon.

‡The acronyms of the regions are as follows: Bahia (BA), Ceará (CE), the Federal District (DF), Espírito Santo (ES), Goiás (GO), Minas Gerais (MG), Rio de Janeiro (RJ) and São Paulo (SP).

system was dipped for 3 min in the conidial suspension after removing the apical sector (about 2 cm). Afterward, the plantlets were transplanted to 1·5 kg plastic pots, with sterile soil and maintained under greenhouse conditions. After transplanting, 3 ml of the conidial suspension was added in the crown area of each plantlet (Reis *et al.*

2005). The experimental plots were made of three 1·5-l pots with three plants each. Air temperature in the greenhouse varied from 24 to 33°C throughout most of the assays, which is within the optimum range of thermal conditions for FOL infection and expression of symptoms (Boix-Ruíz *et al.* 2015). The pathogenicity assays were carried out twice for each individual isolate. The classification of races was made according to the response observed in each differential accession. Disease was assessed 21 days after inoculation using the following disease severity index (DSI): 1 = plant free of symptoms; 2 = plant without wilt symptoms but conspicuous vascular browning is present; 3 = plants showing vascular browning symptoms and wilt symptoms but without leaf yellowing; 4 = plants showing vascular browning and severe wilting associated with the presence of foliar necrosis and chlorosis; 5 = dead plants (Reis *et al.* 2004; Reis and Boiteux 2007). The isolates were classified as pathogenic/virulent only when they were able to induce characteristic vascular browning and wilt symptoms (DSI grade $\geq$ 2·01) in all inoculated plants. At the end of the evaluation, the fungus was re-isolated of symptomatic plants and PCR was carried out to confirm the fungal/race identification.

Molecular characterization of FOL races via molecular markers

Total DNA was individually extracted from all 143 isolates using a modified CTAB plus organic solvents method (Boiteux *et al.* 1999). The extracted DNA from individual isolates was employed as template in PCR assays with the following set of primer pairs (Hirano and Arie 2006): Uni F (5′-ATC ATC TTG TGC CAA CTT CAG-3′) and Uni R (5′-GTT TGT GAT CTT TGA GTT GCC A-3′) with an expected amplicon size ranging from 670 to 672 bp); SP13 F (5′-GTC AGT CCA TTG GCT CTC TC-3′) and SP13 R (5′-TCC TTG ACA CCA TCA CAG AG-3′) with an expected amplicon size of 445 bp; SP23 F (5′-CCT CTT GTC TTT GTC TCA CGA-3′) and SP23 R (5′-GCA ACA GGT CGT GGG GAA AA-3′) with an expected amplicon size of 518 bp; SPR1 F (5′-GAT GGT GGA ACG GTA TGA CC-3′) and SPR1 R (5′-CCA TCA CAC AAG AAC ACA GGA-3′) with an expected amplicon size of 947 bp. This set of primers is able to discriminate all the three FOL races as well as FORL isolates. The PCR reaction (final volume = 15·5 µl) contained 0·5 µl of dNTPs (2·5 mmol l^{-1} each), 1·25 µl buffer 10X (100 mmol l^{-1} Tris-HCl, 500 mmol l^{-1}, pH 8·3), 0·6 µl (50 mmol l^{-1}) $MgCl_2$, 0·2 µl (5 U μl^{-1}) *Taq* DNA polymerase, 5.95 µl Milli-Q® water, 1 µl (0·5 mmol l^{-1}) each of the individual primer pairs and 5 µl of genomic DNA (20 ng). The PCR conditions for all primers were set at the initial denaturation temperature of 94°C for 1 min, followed by 45 cycles of 94°C for 1 min, annealing at 62°C for 1 min and elongation at 72°C for 2 min, with a final extension at 68°C for 7 min (Hirano and Arie 2006). All PCR products were electrophoresed in a 1·5% agarose gel, stained with ethidium bromide and visualized under UV light.

Results

Morphological characterization of the fungal isolates

A total of 143 isolates rendered colonies with conidia and mycelia with morphological characteristics typical of *F. oxysporum*. Colonies on PDA displayed mycelium initially white that gradually turned to white to pale violet. Microconidia were non-septate, oval or elliptical, produced in false heads on short monophialides. Macroconidia were 3–5 septate, most slightly curved, produced from monophialides on branched conidiophores in sporodochia. Chlamydospores were either intercalary or terminal, with either a smooth or rough wall appearance, formed singly or in pairs (Leslie and Summerell 2006). All of them were used in the subsequent tests aiming to confirm their *forma specialis* identity and race classification using the differential set of tomato accessions in combination with the molecular marker system developed by Hirano and Arie (2006).

Reaction of the differential tomato accessions to Brazilian FOL isolates

According to the virulence assays five race 1, twenty-three (23) race 2 and 115 race 3 isolates were identified. Our survey confirmed the presence of FOL race 3 isolates in the States of Espírito Santo, Rio de Janeiro and Bahia, but also uncovered the incidences in the Federal District, Minas Gerais, Goiás, Ceará and São Paulo states (Fig. 1). Race 1 and race 2 isolates prevailed up to early 2000s, after 2002 the isolates were identified as belonging almost exclusively to race 3 (Table 1). The exceptions were two isolates of race 1 (Tianguá–CE and Baldin–MG) and one isolate race 2 (Venda Nova do Imigrante-ES). These results indicated that in recent years the geographical expansion of race 3 FOL in Brazil has been very fast, in 3 years this pathogen has been described in four new States in the country.

Molecular characterization of Brazilian FOL races

Total genomic DNA extracted from this collection of isolates was used to confirm the fungal species, *forma specialis*, and the corresponding physiological race of each of the 143 isolates found in association with vascular wilt of

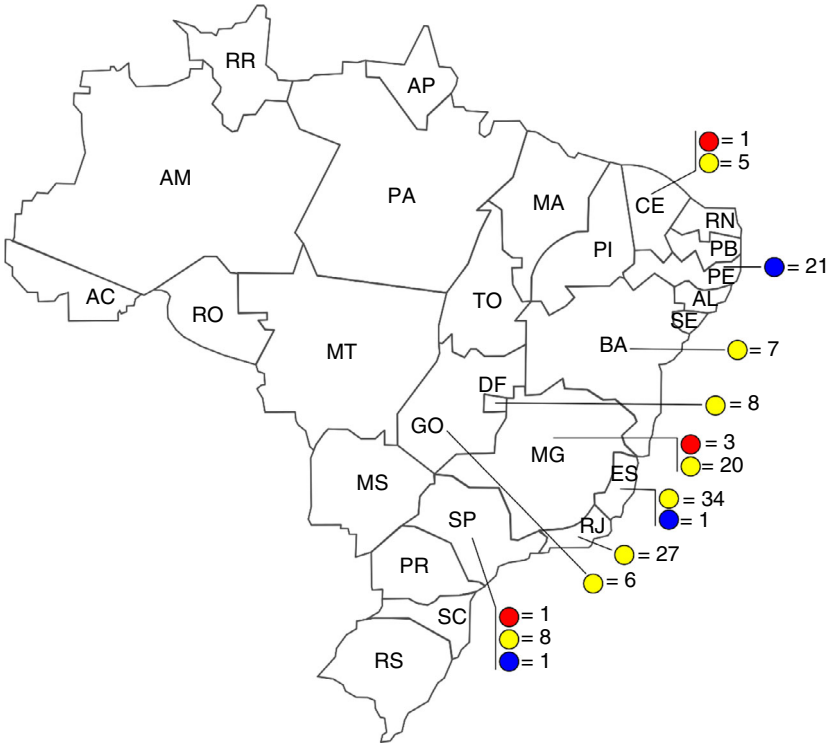

Figure 1 Geographical distribution across major tomato-producing regions in Brazil of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) isolates from distinct physiological races as determined by their ability to infect a set of differential *Solanum* (section *Lycopersicon*). All race 2 isolates displayed amplicon profiles identical to that of the race 1 isolates in assays using the molecular marker system developed by Hirano and Arie (2006). The acronyms of the geographic regions are as follows: Bahia (BA), Ceará (CE), the Federal District (DF), Espírito Santo (ES), Goiás (GO), Minas Gerais (MG), Rio de Janeiro (RJ) and São Paulo (SP) (● Race 1; ● Race 2; ○ Race 3). [Colour figure can be viewed at wileyonlinelibrary.com]

tomatoes using the FOL race-specific molecular marker system developed by Hirano and Arie (2006) (Fig. 2). The employment of this set of race-specific primers (Uni, Sp13, Sp23, and Sprl) was effective in discriminating the 115 race 3 isolates from race 1 and race 2 isolates. However, somewhat surprisingly, this molecular marker system was not able to discriminate the Brazilian race 1 and 2 isolates (compare Table 1 *vs* Fig. 2). All race 1 and 2 isolates displayed a race 1–specific amplicon pattern (*viz.* presence of amplicons with the primer pairs Uni and Sp13, and negative to the others).

Discussion

Pathogenicity assays for determination of FOL races using the complete set of five differential tomato accessions is time-consuming and cumbersome. In addition, these tests should be conducted under controlled environmental conditions that should be suitable for both fungus and host plant to avoid misleading results (Boix-Ruíz *et al.* 2015). For this reason, recent works dealing with the survey and characterization of FOL races are relying exclusively upon molecular markers (Baysal *et al* 2009; Çolak and Biçici 2013; Amaral *et al.* 2013; Debbie *et al.* 2018). However, due to some observed discrepancies, the combination of both pathogenicity tests and molecular marker assays is considered the most robust and consistent strategy for race discrimination (Boix-Ruíz *et al.* 2015). In addition, analyses done exclusively with molecular markers could miss important and useful information from the tomato breeding standpoint, including the potential emergence of novel pathogen variants especially FOL isolates displaying the ability to breakdown resistance of either *I*–3 or *I*–7 genes.

In this context, the present work was carried out by combining pathogenicity tests and molecular marker assays and it represents thus far the most comprehensive analysis of the race variability of FOL isolates associated with tomato fusarium wilt in Neotropical areas. The pathogenicity/virulence tests using the complete set of five differential tomato accessions allowed us to catalogue the presence in the collection of five race 1, twenty-three (23) race 2 and 115 race 3 isolates collected from 1990 to 2019 across major tomato-producing regions in the

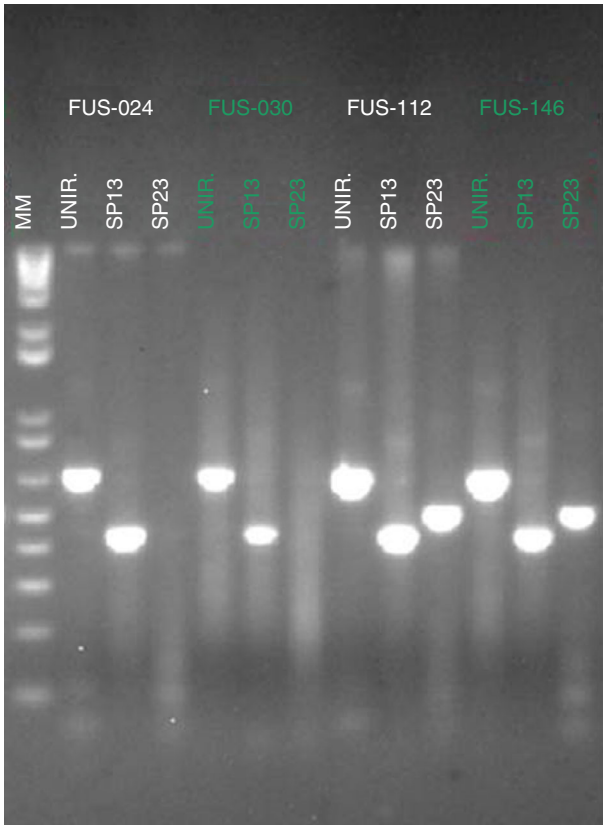

Figure 2 PCR amplicon profiles of a subset of Brazilian isolates of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) obtained with the following primer pairs (and their respective amplicon sizes): Uni (672 bp), Sp13 (445 bp) and Sp23 (518 bp). The FOL isolate Fus–024 (collected in Belém S. Francisco, Pernambuco State in 1991) was classified as physiological race 2, whereas the isolate Fus–030 (collected in Uberlândia, Minas Gerais State in 1992) was classified as race 1 after inoculation assays employing a set of differential tomato (*Solanum* section *Lycopersicon*) accessions. However, both Fus–024 and Fus–030 displayed race 1 amplification profiles. The isolates Fus–112 (collected in Venda Nova do Imigrante, Espirito Santo in 2004) and Fus–146 (collected in Domingos Martins, Espírito Santo State in 2006) were classified as FOL physiological race 3 and displayed a typical race 3 amplification profile. MM = molecular marker (100 bp ladder). [Colour figure can be viewed at wileyonlinelibrary.com]

country. The majority of these samples was obtained from fresh-market tomato fields with only three isolates (Fus–484, Fus–485 and Fus–486) obtained from processing tomatoes. Analyses employing the molecular marker system for differentiation of FOL races developed by Hirano and Arie (2006) were extremely precise in distinguishing all 115 race 3 isolates, displaying a perfect correlation between pathogenicity/virulence tests and PCR amplicon patterns obtained with the primer pairs Uni, Sp13, Sp23 and Sprl (Table 1, Fig. 2). Therefore, this marker system can be recommended for fast and unambiguous diagnosis of isolates from this physiological race. However, this molecular marker system was not efficient in differentiating the Brazilian races 1 and 2. Isolates of both races displayed (without exceptions) identical amplicon pattern corresponding to that described for the cosmopolitan race 1 isolates (Hirano and Arie 2006).

The emergence of new races from existing variants of a pathogen, in some circumstances, is favoured by the intensive use of cultivars with monogenic resistance (Robinson 1980). Our results indicated that this type of microevolutionary event may have occurred endemically with the Brazilian FOL race 2 isolates. In the 1930s and 1940s, the most common cultivars were small-fruited types. These cultivars were originated mainly from seeds imported by immigrants from Italy and Japan, which was the most likely original vehicle for introduction of the cosmopolitan race 1 into Brazil (Melo *et al.* 2009). This scenario changed in the early 1940s with the selection of cultivars with large fruits from the 'Santa Cruz' group (e.g. 'Santa Cruz CAC', 'Kada' and 'Yokota'), which were initially introduced in São Paulo State and soon they were widely accepted in this major producing region of the country (Melo *et al.* 2009). In fact, up to the early 1990s the open-pollinated cultivars belonging to the 'Santa Cruz' varietal group (especially the cv. Santa Clara) had the leadership in the fresh-market tomato segment. The majority of the original cultivars from the 'Santa Cruz' group displayed high levels of susceptibility to FOL race 1 isolates. The release of improved cultivars such as 'San Antonio', 'Miguel Pereira' and 'Angela' (Nagai 1993) with resistance to FOL race 1 (due to the presence of the *I*–1 gene) had major commercial impact and they became market leaders in São Paulo and Rio de Janeiro States, spreading very rapidly to other tomato production zones of the country (Melo *et al.* 2009). Race 2 isolates were initially described in tomato-producing areas in São Paulo with a predominance of cultivars from 'Santa Cruz' group (with only domestic seed production), minimizing, therefore, the chances of introducing novel FOL isolates from abroad. This peculiar situation reinforces the hypothesis of a putative endemic origin of the Brazilian race 2 isolates. The initial report of race 2 in São Paulo State could explain the occurrence of genetically similar isolates across other geographical regions as observed here in a subset of isolates from Pernambuco State (see Table 1 and Fig. 2). FOL is a seed-transmissible pathogen and seeds (and often seedlings) of the major 'Santa Cruz' cultivars (both resistant and susceptible to race 2) were mainly produced in São Paulo State and then distributed throughout the country. In this scenario, two working hypotheses were proposed to explain the occurrence of race 2 in Brazil: (i) high frequency of

mutation from race 1 to race 2 and (ii) this mutation occurred in the direction of increasing the virulence profile and aggressiveness of race 1 isolates previously existing across the Brazilian tomato–producing regions (Tokeshi and Galli 1966). Our analysis using the race-specific molecular marker system developed by Hirano and Arie (2006) with Brazilian race 2 isolates displaying typical amplicon profile of the cosmopolitan race 1 isolates gives support to both hypotheses. Reinforcing this view, Amaral *et al.* (2013) carried out cluster analyses with a sample of FOL races from Brazil, employing combined information derived from random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) and restriction fragment length polymorphism of intergenic space (RFLP-IGS) region. They observed that Brazilian race 1 and race 2 isolates were clustered into the same group, whereas race 3 isolates were placed in a distinct cluster. High levels of genetic identity (ranging from 93 to 100%) between Brazilian race 1 and 2 isolates were also observed by Kuramae and Souza (2002) employing information from RAPD markers, 5.8S rDNA gene, and from ITS1 and ITS2 (internal transcribed spacer). Another possible explanation for the lack of molecular polymorphisms among Brazilian race 1 and race 2 isolates is that all race 2 isolates are, in fact, race 1 isolates virulent to tomato lines carrying the *I*–2 gene as previously reported by Mes *et al.* (1999). The genomic analysis of the *Avr* gene repertoire (Lievens *et al.* 2009; Cao *et al.* 2018) of these Brazilian isolates will help to clarify this point.

In the early 1990s, the long-shelf life hybrids were introduced from abroad and they were widely adopted in the country (Melo *et al.* 2009). After that, Brazil became a major importer of tomato seeds. Most of the long-shelf life hybrids available in the Brazilian market were resistant to both race 1 and race 2. Even though previous misleading reports indicated the presence of FOL race 3 in Brazil (Tokeshi and Galli 1966; Tokeshi *et al.* 1966; Tokeshi and Noguez 1974), the first official record of this tomato pathogen in the country was done in the early 2000s in long-shelf life hybrids (harbouring the *I* and *I*–2 genes) in the States of Espírito Santo (Reis *et al.* 2005), Rio de Janeiro (Reis and Boiteux 2007) and Bahia (Barboza *et al.* 2013). These peculiar changes in the Brazilian tomato seed market may explain the absence of PCR patterns of the cosmopolitan race 2 isolates (Hirano and Arie 2006) in our samples (indicating either absence or no significant introduction of this race from abroad) as well as our observation that race 1 and 2 isolates prevailed up to early 2000s, after that time the large majority of the isolates was classified as race 3.

Our survey indicated a recent and dramatic dispersion of FOL race 3 isolates across many new regions of the country. Soon afterwards the initial reports (Reis *et al.* 2005; Reis and Boiteux 2007; Barboza *et al.* 2013), new race 3 isolates were collected between 2012 and 2015 in the region of Zona da Mata of Minas Gerais State (Southeast region). More recently, novel race 3 samples were also obtained in commercial fields from the States of Goiás (Center–East), São Paulo (Southeast), Ceará (Northeast) and in the Federal District (Center–East region) (Table 1 and Fig. 1). This fast and extensive dissemination of race 3 in Brazil suggests its introduction and nationwide dispersion of the race 3 isolates via contaminated propagative material (Reis *et al.* 2005). Interestingly, the majority of the FOL isolates collected after the 2003 were identified as race 3, which coincides with the first formal field reports of this race in the country (Reis *et al* 2005; Reis and Boiteux 2007). This introduction of race 3 in the country was followed by a relatively fast replacement by the growers in some producing regions of susceptible cultivars by the ones carrying either the *I*–3 or *I*–7 resistance genes (Gonçalves *et al.* 2018). It is noteworthy that the perfect correlation between pathogenicity tests and PCR amplicon profiles may also indicate either few events of race 3 invasion (resulting in low overall race variability) or the introduction of contaminated seeds from countries were the polymorphisms associated with the PCR amplicon profiles described by Hirano and Arie (2006) were preserved.

Similar to our observations in relation to the Brazilian race 1 and race 2 isolates, some discrepancies have been also observed between the pathogenicity tests and the PCR-based marker system described the Hirano and Arie (2006) in the literature (Baysal *et al* 2009; Çolak and Biçici 2013; Boix-Ruíz *et al.* 2015). For this reason, the development and/or validation of novel or distinct race-specific marker systems are necessary to allow a precise discrimination of the FOL races under Brazilian conditions. A group of small, cysteine-rich proteins named as SIX (secreted into the xylem) are effectors that play a crucial role in host colonization, symptom expression (Houterman *et al.* 2007) and a subset of them is directly associated with the avirulence/virulence profile of the FOL races (Lievens *et al.* 2009; Cao *et al.* 2018). Once detected by the tomato surveillance/defence machinery (that includes the *I*, *I*–2, *I*–3 and *I*–7 resistance genes), a subset of these SIX proteins are converted into avirulence factors (Lievens *et al.* 2009; Cao *et al.* 2018). For example, the gene products of *Six*4 (=*Avr*1), *Six*3 (=*Avr*2) and *Six*1 (=*Avr*3) are able to activate the resistant reaction mediated by *I*, *I*–2 and *I*–3, respectively. This strong functional correlation between this subset of *Six* genes and FOL virulence/avirulence profile makes a marker system derived from this genomic information a very powerful tool for precise identification of physiological races of this

pathogen (Lievens *et al.* 2009). In fact, the *Six*4 gene has been used to identify isolates of FOL race 1, the *Six*3 polymorphism can be exploited to differentiate isolates of FOL race 2 and race 3 (Lievens *et al.* 2009), whereas the combination of *Six*3 (=*Avr*2) and *Six*5 genes triggers the resistant reaction under control of the *I*–2 gene (Cao *et al.* 2018). Therefore, additional assays employing the set of primers derived from the SIX-series genes may be a robust tool to categorize the Brazilian races of FOL in future works.

In conclusion, the characterization of this collection of isolates demonstrates the global variability of this pathogen and provides indications that different evolutionary mechanisms may be acting to select new FOL races and variants in the different tomato agroecosystems across different geographical regions in Brazil and globally. The results from our study confirm the polyphyletic nature of a subset of FOL isolates and provide useful information for guiding tomato breeding strategies aiming for the development of cultivars with both durable and phenotypically stable resistance.

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Conflict of Interest

The authors declare no competing interests.

Author contributions

Hélcio Costa, Ailton Reis, Amanda M. Gonçalves and Fabiana H. S. Ribeiro collected and carried out the initial morphological characterization of the fungal isolates. Ailton Reis, Leonardo S. Boiteux and Maria Esther Fonseca planned and coordinated the laboratories involved in the research. Amanda M. Gonçalves, Cléia Cabral and Fabiana H. S. Ribeiro performed the bioassays and Lab bench work. This work is part of the research for Amanda Melo's doctoral thesis. All authors contributed to the writing and revision of the manuscript.

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